



**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2014

Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease

Gutiérrez, Ana ; Scharl, Michael ; Sempere, Laura ; Holler, Ernst ; Zapater, Pedro ; Almenta, Isabel ;
González-Navajas, José M ; Such, José ; Wiest, Reiner ; Rogler, Gerhard ; Francés, Rubén

Abstract: **OBJECTIVE:** The aetiology of Crohn's disease (CD) has been related to nucleotide-binding oligomerisation domain containing 2 (NOD2) and ATG16L1 gene variants. The observation of bacterial DNA translocation in patients with CD led us to hypothesise that this process may be facilitated in patients with NOD2/ATG16L1-variant genotypes, affecting the efficacy of anti-tumour necrosis factor (TNF) therapies. **DESIGN:** 179 patients with Crohn's disease were included. CD-related NOD2 and ATG16L1 variants were genotyped. Phagocytic and bactericidal activities were evaluated in blood neutrophils. Bacterial DNA, TNF, IFN, IL-12p40, free serum infliximab/adalimumab levels and antidrug antibodies were measured. **RESULTS:** Bacterial DNA was found in 44% of patients with active disease versus 23% of patients with remitting disease ($p=0.01$). A NOD2-variant or ATG16L1-variant genotype was associated with bacterial DNA presence (OR 4.8; 95% CI 1.1 to 13.2; $p=0.001$; and OR 2.4; 95% CI 1.4 to 4.7; $p=0.01$, respectively). This OR was 12.6 (95% CI 4.2 to 37.8; $p=0.001$) for patients with a double-variant genotype. Bacterial DNA was associated with disease activity (OR 2.6; 95% CI 1.3 to 5.4; $p=0.005$). Single and double-gene variants were not associated with disease activity ($p=0.19$). Patients with a NOD2-variant genotype showed decreased phagocytic and bactericidal activities in blood neutrophils, increased TNF levels in response to bacterial DNA and decreased trough levels of free anti-TNF. The proportion of patients on an intensified biological therapy was significantly higher in the NOD2-variant groups. **CONCLUSIONS:** Our results characterise a subgroup of patients with CD who may require a more aggressive therapy to reduce the extent of inflammation and the risk of relapse.

DOI: <https://doi.org/10.1136/gutjnl-2012-303557>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-92860>

Journal Article

Accepted Version

Originally published at:

Gutiérrez, Ana; Scharl, Michael; Sempere, Laura; Holler, Ernst; Zapater, Pedro; Almenta, Isabel; González-Navajas, José M; Such, José; Wiest, Reiner; Rogler, Gerhard; Francés, Rubén (2014). Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease. *Gut*, 63(2):272-280.

DOI: <https://doi.org/10.1136/gutjnl-2012-303557>

Title: Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease.

Authors: Ana Gutiérrez¹, Michael Scharl², Laura Sempere¹, Ernst Holler³, Pedro Zapater^{1,4}, Isabel Almenta¹, José M González-Navajas^{1,4}, José Such^{1,4}, Reiner Wiest⁵, Gerhard Rogler², Rubén Francés^{1,4}.

Affiliations:

¹Servicio de Medicina Digestiva, Hospital General Universitario de Alicante, Alicante, Spain; ²Division of Gastroenterology and Hepatology, University Hospital Zürich, Zürich, Switzerland; ³Department of Internal Medicine I, University of Regensburg, Germany; ⁴CIBERehd, Instituto de Salud Carlos III, Madrid, Spain; ⁵University Clinic for Visceral Medicine, Department Gastroenterology, Inselspital, Bern, Switzerland

Corresponding author: Rubén Francés, PhD. CIBERehd - Hospital General Universitario de Alicante. Avda. Pintor Baeza 12, Alicante 03010, Spain. Tel: +34 965 913 928. Fax: +34 965 913 922. E-mail: frances_rub@gva.es

Keywords: Crohn's disease; bacterial DNA; NOD2; ATG16L1; TNF-alpha, biological therapy.

Grant support: This work has been supported in part by grants CP05/0005 and PI10/0340, Instituto de Salud Carlos III, Madrid, Spain, and with funding obtained from Fundación FCVI-HGUA, Alicante, Spain. MS and GR were supported by an educational grant from Essex Chemie, Switzerland, a research credit from the University of Zurich to MS, a grant from the Broad Medical Research Program to GR (Grant No. IBD-0241R1), a grant from the Swiss National Science Foundation (Grant No.: 310030-120312) to GR and the Zurich Centre for Integrative Human Physiology.

Electronic Word count: 4138

Authors' contribution

AG: patients' inclusion and manuscript writing

AG, LS, IA: diagnosis, treatment and follow-up of patients

MS, EH: NOD2 and ATG16L1 genotyping

PZ, JMG, RF: experimental data, acquisition and analysis

JS, RW, GR: critical revision and clinical considerations

RF: study concept and design, manuscript writing

Conflict of interests:

Authors have no conflicts of interests

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non-exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its licensees to permit this article (if accepted) to be published in Gut editions and any other BMJ PGL products to exploit all subsidiary rights, as set forth in our licence.

Abstract

Objective: The aetiology of Crohn's disease (CD) has been related to NOD2 and ATG16L1 gene variants that disrupt an autophagy-dependent antibacterial pathway. The observation of bacterial-DNA translocation in the blood of CD patients led us to hypothesize that an impaired immune response may facilitate this process in patients with NOD2/ATG16L1-variant genotypes and affect the efficacy of anti-TNF therapies.

Design: 179 patients with Crohn's disease were included. CD-related NOD2 and ATG16L1 variants were genotyped. Phagocytic and bactericidal activities were evaluated in blood neutrophils. Bacterial-DNA, TNF- α , IFN- γ , IL-12p40, free serum infliximab/adalimumab levels and anti-drug antibodies were measured.

Results: Bacterial-DNA was found in 44% of active patients vs. 23% of patients in remission ($p=0.01$). A NOD2 or ATG16L1-variant genotype was significantly associated with the presence of bacterial-DNA (Odds ratio (OR) 4.8; 95%CI 1.1-13.2; $p=0.001$, and OR 2.4; 95%CI 1.4-4.7; $p=0.01$, respectively). This OR was 12.6 (95%CI 4.2-37.8; $p=0.001$) for patients with a double-variant genotype. Presence of bacterial-DNA in blood was independently associated with disease activity (OR 2.6; 95%CI 1.3-5.4; $p=0.005$). Single and double-gene variants were not directly associated with disease activity ($p=0.19$). Patients bearing a NOD2-variant genotype showed significantly decreased phagocytic and bactericidal activities in blood neutrophils, increased TNF- α levels in response to bacterial-DNA and significantly decreased trough levels of free anti-TNF- α . Interestingly, the proportion of patients on an intensified biological therapy was significantly higher in the NOD2-variant groups.

Conclusion: The results point to the identification of a patient subgroup that might require more aggressive therapies to reduce inflammation, the risk of bacterial-DNA translocation and relapse.

Electronic word count: 253

Summary box

What is already known about this subject?

- Genetic susceptibility and the immunological interaction between the host and commensal flora are implicated in the aetiology of Crohn's disease.
- Gut bacterial DNA translocation into blood is a frequent event in patients with Crohn's disease.
- Relapse occurs during the course of the disease in patients with Crohn's disease, even on immunosuppressive and/or biological maintenance treatments.
- The efficacy of different therapeutic schemes using biologics to treat patients with Crohn's disease is under discussion.

What are the new findings?

- NOD2 and ATG16L1 genetic variants are independent risk factors for bacterial DNA translocation in Crohn's disease.
- Bacterial DNA translocation is directly associated with disease activity and increases the risk of relapse at 6 months.
- Patients with a NOD2 variant genotype showed significantly decreased phagocytic and bactericidal activities, increased serum TNF-alpha levels in response to bacterial-DNA and significantly decreased levels of free serum anti-TNF- α .
- *In vitro* *E. coli* DNA-stimulated neutrophils from patients with a NOD2 variant genotype show lower serum levels of anti-TNF-alpha, suggesting faster drug consumption

How might it impact clinical practice in the foreseeable future?

- Our results identify a subgroup of patients with an increased risk of bacterial DNA translocation and, very likely, an increased risk of relapse.
- Classical anti-TNF-alpha therapy regimens are likely to decrease their efficacy in controlling the risk of flare-up in these patients who might require more aggressive therapeutic programs to reduce inflammation and episodes of bacterial DNA translocation.

Introduction

The understanding of the pathogenesis of Crohn's disease (CD), one of two major forms of inflammatory bowel disease affecting the population worldwide (1;2), has experienced enormous progress in recent years. Its aetiology is partially explained by a disturbance of the interaction between the host and intestinal microorganisms (3-6). Accordingly, the gut epithelial barrier plays a crucial role in regulating the uptake of luminal antigens and in controlling intestinal immune responses to luminal bacteria. Recently, we have reported evidence of bacterial DNA (bactDNA) translocation into the blood of CD patients. The associated innate immune response, which is partially affected by nucleotide-binding oligomerization domain containing 2 (NOD2) gene status (7;8), suggests either a dysfunction in the epithelial cell layer, an intestinal bacterial overgrowth or the combination of both of these adverse events.

NOD2 encodes an intracellular receptor for the presence of muramyl dipeptide (MDP), a common component of the bacterial cell wall (9-11) and has been identified as a prominent CD susceptibility gene (12). However, genome-wide association studies have increased the number of genes that may potentially modulate CD up to 71 loci (13) (14). Among them, the autophagy gene ATG16L1, is very prominent (15-17). ATG16L1 is implicated in the modulation of autophagy, an important cell survival mechanism also involved in key aspects of the innate immune response to microorganisms (18). The CD-associated ATG16L1 variant, T300A, has been reported to result in an autophagy defect (15;19), suggesting a role for autophagy, and ATG16L1 in particular, in the pathogenesis of CD.

Recently, ATG16L1 and NOD2 have been reported to interact in an autophagy-dependent antibacterial pathway in response to MDP that is disrupted by specific CD-associated mutations (20). We have observed that CD patients with a mutated NOD2 genotype cannot properly respond to bactDNA translocation (8). On the other hand, increasing evidence has been reported linking phagocytosis, a primary mechanism of host defence against bacterial products, with the autophagy machinery (21;22). Therefore, we hypothesize that the presence of bactDNA in the blood of CD patients may be associated with functional impairment by the autophagy-related CD-associated gene mutation, further complicating the disease outcome.

The aim of the present study was to determine whether the presence of NOD2 and ATG16L1 mutations confer susceptibility to an impaired phagocytic activity and increased rates of bactDNA translocation into the blood of CD patients.

Patients and methods

Patients.

One hundred-seventy-nine consecutive patients diagnosed with CD and controlled in the IBD Unit of the Hospital General Universitario de Alicante who accepted to participate in the study were included. The diagnosis of CD was established according to standard clinical, endoscopic, histological and radiographic criteria (23). Patients who had received antibiotics in the previous 2 weeks were not considered for inclusion. All patients were classified according to the Montreal classification (24) and stratified according to the Crohn's Disease Activity Index (CDAI). All included patients received diaries to record symptoms one week prior to inclusion and sample collection. Intensified therapy with biologics was defined either by an increased dose or an increase in the frequency of infusions versus dosing or schedule upon start of treatment. Twenty-five healthy controls were also included in the study. All patients and controls were Caucasian of Mediterranean ethnicity. Blood samples from patients and healthy donors were obtained for routine haematological and biochemical studies and inoculated in aerobic and anaerobic blood culture bottles, 10mL each. Simultaneously, two separate blood samples were inoculated under aseptic conditions in rubber-sealed sterile Vacutainer SST II and K3E tubes, respectively (BD Diagnostics, Belgium) that were never exposed to free air.

Patients were followed-up for 6 months to evaluate the incidence of relapse. Although "relapse" strictly refers to the clinical situation coming from remission, we wanted to evaluate whether patients would turn into an active disease form in the next 6 months after bactDNA detection, no matter their CDAI status at admission. In the case of patients with a CDAI > 150 at admission, the flare was controlled in all cases within the subsequent two weeks. The Ethics Committee of the hospital approved the study protocol. All controls and patients gave informed consent prior to inclusion in the study.

Identification of bactDNA fragments and genotyping of CD-associated genes.

Genomic DNA was isolated from 5×10^6 cells with the QIAmp DNA Blood Minikit (Qiagen, Hilden, Germany). BactDNA was identified by running broad-range PCR followed by partial nucleotide sequencing of a conserved region of 16SrRNA gene, as previously described (25). The three common NOD2/CARD15 allelic variants at SNP-8 (R702W, rs2066844), SNP-12 (G908R, rs2066845) and SNP-13 (L1007finsC,

rs2066847) were evaluated as previously described (26). The ATG16L1 variation rs2241880 (T300A) was genotyped by TaqMan technology (Applied Biosystems) using commercially available TaqMan SNP Genotyping Assays and TaqMan Genotyping Master Mix on a 7900HT Fast Real-Time PCR System using SDS 2.2 Software (Applied Biosystems). A variant NOD2 genotype (varNOD2) was defined as carrying any of the three variants studied either in homozygosis or heterozygosis. A variant ATG16L1 genotype (varATG16L1) was defined as carrying the rs2241880 variation either in homozygosis or heterozygosis. All genotyping results were assessed twice. The evaluators were not aware of either the patient's disease-status or each other's genotype results. A Hardy-Weinberg test was performed as a quality control measure in controls. No missing genotypes were present.

Phagocytic and bactericidal assays

The phagocytic activity of neutrophils that ingest fluorescein-isothiocyanate (FITC)-labelled opsonized *E. coli* and the bactericidal activity of neutrophils that oxidize the fluorogenic substrate dihydrorhodamine DHR123 to rhodamine R123 (ability to generate reactive oxygen species) were determined using the Phagotest and Bursttest Kits (Orpegen Pharma, Heidelberg, Germany) following the manufacturer's instructions. Cells were quantified by flow cytometry using a FacsCanto (Becton Dickinson, Heidelberg, Germany) and the data were processed using the FacsDiva 12.0 software (Becton Dickinson).

Serum cytokine and free anti-TNF- α levels. Presence of anti-drug antibodies.

Enzyme-linked immunosorbent assays (ELISAs) were carried out to measure TNF- α , IFN- γ , IL-12p40 (R&D Systems, Minneapolis, MN), free infliximab and adalimumab levels and to detect anti-drug antibodies (Matriks Biotek, Ankara, Turkey) according to the manufacturers' instructions. All samples were tested in triplicate and read in a Sunrise Microplate Reader (Tecan, Männedorf, Switzerland). The detection limit for each cytokine assay varied between 2–5 pg/mL and between 10-30ng/mL in the case of free anti-TNF- α kits. The presence of anti-drug antibodies was evaluated by a cut-off value estimated by multiplying the optical density (OD) of the zero standard by 3, as indicated by the manufacturers. Samples were considered positive when the ratio sample OD/zero standard OD was higher than 3.

Isolation and culture of human PMN cells.

To study *in vitro* the relationship between free anti-TNF- α levels in neutrophils from patients with different NOD2/ATG16L1-combined genotypes, PMNs from peripheral blood samples were isolated with PolymorphPrepTM (Axis-Shield PoC, Oslo, Norway). After isolation of PMNs, cells were washed twice with freshly made PBS at 4°C. Cell viability was evaluated by trypan blue (Sigma, Madrid, Spain). Cells were resuspended in phenol red-free RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% human serum AB (BioWhittaker, Walkersville, MD). Neutrophils (1×10^6 cells/well) were incubated with 200 ng of *E. coli*-DNA. Half of the supernatant volumes were collected at 24 hours and TNF- α and annexin V levels measured by ELISA. Cells were then treated with Infliximab 100ug/mL/ 10^6 cells (Infliximab was kindly provided by Merck Sharp and Dohme of Spain MSD, Madrid, Spain). Supernatants were collected at 48 hours to determine free TNF- α , anti-TNF- α and annexin V levels. Total endogenous annexin V levels were evaluated as an indirect measurement of apoptotic activity in neutrophils using a human Annexin V ELISA kit from Promokine (Heidelberg, Germany), according to manufacturer's instructions.

Statistical Analysis

Continuous variables are reported as the mean \pm standard deviation and categorical variables as frequency or percentages. The Kolmogorov-Smirnov test was used to test the normality of the distribution of continuous variables. Statistical differences were analysed using the chi-squared test for categorical data followed by the *post-hoc* Bonferroni correction for multiple comparisons. Quantitative data showing normal distribution were analysed using the ANOVA test followed by the *post-hoc* Bonferroni correction for multiple comparisons. Quantitative data showing non-normal distribution were analysed using the Mann-Whitney U test or Kruskal-Wallis test followed by pairwise comparisons using the Mann-Whitney U test with the *post-hoc* Bonferroni correction for multiple comparisons. Bivariate correlations were analysed using the Spearman test. Logistic regression analysis was used to determine the association between NOD2/ATG16L1 genotypes and bactDNA concentration or CDAI. We first examined clinical and experimental variables by univariate analysis, and then by multivariate analysis including significant factors at $P < 0.10$ in univariate analysis. Results of logistic regression are reported as odds ratio (OR) and 95% confidence interval (95%CI). All reported p-values are two-sided, and p-values less than 0.05

were considered to indicate significance. All analyses were performed using IBM SPSS Statistics 19.

Results

Patient characteristics

The clinical and analytical characteristics of patients included in the study are detailed in Table 1, stratified according to the Crohn's Disease Activity Index (CDAI). No statistically significant differences were observed between groups regarding age, gender, smoking habit or disease classification by Montreal criteria. Active patients showed higher ESR, CRP and lower haemoglobin and albumin levels than patients in remission. Steroid treatment was significantly more frequent in active patients. Among healthy controls, mean age was 32 ± 11 years, male/female ratio was 12:8, and 35% of them were smokers. No differences in demographic and analytical parameters between controls and patients in remission were observed. All patients and controls included in the study had negative blood microbiological cultures.

NOD2 / ATG16L1 genotypes and serum bactDNA distribution in CD patients.

The distribution of genotypes and allelic frequencies in CD patients and controls are shown in Table 2. All variants were found to be in the Hardy-Weinberg equilibrium in the controls. Overall, 69% of patients in remission and 76.4% of active patients showed at least one of the CD-associated gene variants. A NOD2 variant, either alone or combined with the ATG16L1 variant, was present in 56 (41.2%) patients in remission and in 18 (41.8%) active patients. The ATG16L1 variant, either alone or combined with a NOD2 variant, was present in 82 (60.2%) patients in remission and in 24 (55.8%) active patients. Figure 1A shows the distribution of NOD2 and ATG16L1 variants among active CD patients and those in remission. The distribution of the genotypes under study was not statistically significant between both groups ($p=0.90$).

From the overall series of patients, 51 out of 179 (28.5%) showed the presence of bactDNA in blood samples. By groups, 19 out of 43 clinically active patients (44.2%) and 32 out of 136 patients in remission (23.5%) showed bactDNA ($p=0.01$). The identified bacterial species included: *Escherichia coli* (n=21), *Staphylococcus aureus* (n=12), *Klebsiella pneumoniae* (n=7), *Streptococcus pneumoniae* (n=5), *Shigella spp.* (n=3), *Enterococcus faecalis* (n=2) and *Campylobacter freundii* (n=1). No statistical differences in bacterial species were observed between the studied genotypes. As expected, bactDNA was absent in the blood of healthy controls. A univariate analysis revealed that the presence of bactDNA in the blood, among

others, was significantly associated with disease activity with an odds ratio (OR) 2.6 (95% confidence interval (95%CI) 1.3-5.4; $p=0.005$) whereas single and double-gene mutations were not directly associated with disease activity ($p=0.19$). As expected, the use of steroids and infliximab were also significantly associated with disease activity, as they are a usual therapeutic first approach on a flare-up (Supplementary Table 1). A multivariate analysis including variables showing a p value <0.1 on the univariate analysis is detailed in Table 3A. Of note, bactDNA remained significantly and independently related to disease activity.

BactDNA was present in 5/47 (10.6%) wtNOD2/wtATG16L1 patients, 8/26 (30.7%) varNOD2/wtATG16L1 patients, 9/57 (15.5%) wtNOD2/varATG16L1 patients and 29/48 (60.4%) varNOD2/varATG16L1 patients. Figure 1B displays the percentage of bactDNA presence in each studied genotype, distributed by CDAI. The percentage of patients with bactDNA was significantly increased in the varNOD2 genotype groups. Unlike the double wild-type genotype, single NOD2 or ATG16L1 variants were significantly associated with the presence of bacterial DNA on the univariate analysis (OR 4.8; 95%CI 1.1-13.3; $p=0.03$, and OR 2.4; 95%CI 1.46-4.5; $p=0.05$, respectively). This effect was more pronounced in patients with a double variant genotype (OR 12.7; 95%CI 4.2-37.8; $p=0.001$) (Supplementary Table 2). All studied gene variants, as well as disease activity, remained significantly and independently associated with the presence of bactDNA on the multivariate analysis (Table 3B).

No differences among the three examined NOD2 allelic variants were observed for CDAI or any other clinical characteristics of varNOD2 patients. No statistically significant differences were observed, either, between patients stratified by genotype or by presence of bactDNA, although CRP, total WBC and CDAI were higher in patients with bactDNA (data not shown).

Forty-three patients relapsed during this period (24%): twenty out of 51 patients with bactDNA at inclusion (40%) vs. 23 out of 128 patients without bactDNA (18%) ($p=0.01$). BactDNA in the blood, among others, was significantly associated with an increased risk of flare-up at 6 months on the initial univariate analysis, with an OR 3.32 (95%CI 1.5-7.3; $p=0.003$) (Supplementary Table 3). The multivariate analysis revealed bactDNA presence as the only independent factor associated with relapse at 6 months (Table 3B). Thirty-three patients (18.4%) received short-term courses of

oral antibiotics during the 6-month follow-up period due to non CD-related causes with no statistically significant effect on the risk for relapse among patients, either stratified by genotype or by the presence of bactDNA at admission. There was no statistical association either between bactDNA presence at admission and the subsequent need for antibiotics, complications such as surgery or re-admissions during the follow-up period among the different genotype subgroups.

Immunological activity in response to bactDNA in patients with different NOD2/ATG16L1 genotypes.

We evaluated the pro-inflammatory cytokine response to the presence of bactDNA in the serum of CD patients, stratified by genotype. The results are shown in Table 4. No differences were observed between patient groups without bactDNA. The pro-inflammatory activity was significantly increased in patients with bactDNA as compared with that observed in patients without bactDNA, irrespective of genotype. However, patients with bactDNA and a variant NOD2 genotype, either alone or combined with a variant ATG16L1 genotype, showed a further statistically significant increase as compared with wild-type NOD2 genotype subgroups. Furthermore, the highest pro-inflammatory levels were obtained in serum from patients carrying a combined NOD2/ATG16L1 double-variant genotype. An ANOVA analysis of pro-inflammatory cytokines including different variables and their combinations revealed that bactDNA and the interaction between bactDNA and genotype were the only statistically significant variables (data not shown).

Phagocytic and bactericidal activities were evaluated in blood neutrophils treated with a fluorochrome-labelled *E. coli* from the first 12 patients in remission without bactDNA of each genotype-stratified subgroup (n=48). As shown in Figure 2, phagocytic and bactericidal activities were significantly decreased in the neutrophils of patients with varNOD2/wtATG16L1 and varNOD2/varATG16L1 genotypes, with no differences between these two groups. To investigate whether these results were affected by ongoing treatment, phagocytic and bactericidal activities were also evaluated in healthy controls without bactDNA carrying NOD2 (n=3) and ATG16L1 (n=5) variants or wild-type (n=10). As in patients, these activities were decreased in healthy donors carrying any NOD2 variant as compared with ATG16L1 variant and wild-type genotypes (data not shown).

Intensified anti-TNF- α therapy is more frequent in patients with mutated NOD2/ATG16L1-combined genotypes.

Next, we evaluated whether these combined genotypes had an impact on anti-TNF- α therapy schedules. Fifty-two (29%) out of the 179 patients were on anti-TNF- α therapy at inclusion, either combined with immunosuppressive treatment or not, 30 on infliximab and 22 on adalimumab (see Table 1). Figures 3A and 3B show the percentage of patients on anti-TNF- α therapy and, of those, the percentage of patients on an intensified schedule of anti-TNF- α therapy, either stratified by genotype or by the presence of bactDNA. As detailed, the proportion of patients on intensified therapy was higher in the groups with either a varNOD2/wtATG16L1 or a varNOD2/varATG16L1 genotype. No statistically significant differences were observed in the percentage of smokers, the time from diagnosis or the time from initiation of anti-TNF- α therapy between intensified and non-intensified patients, all of which could affect the need for intensification (Supplementary Table 4). Twenty percent of patients receiving anti-TNF- α therapy showed bactDNA in blood, of which 50% were on an intensified drug schedule. By contrast, only 33% of patients without bactDNA received intensified treatment ($p=ns$). No differences were observed for the distribution of each anti-TNF- α among patients, either grouped by genotype or by the presence of bactDNA.

Free anti-TNF- α levels were measured in the serum of patients receiving therapy with biologics and stratified according to their genotype and the presence of bactDNA. Results were restricted to samples that were negative for the presence of anti-drug antibodies ($n=48$) and are shown in Figure 3C. Significantly decreased levels of free anti-TNF- α were observed in the varNOD2/wtATG16L1 and varNOD2/varATG16L1 genotype-based subgroups, despite showing a significant number of patients on an intensified schedule. The presence of bactDNA further decreased the levels of free serum anti-TNF- α , although the number of individuals was too small to report statistics. Since interactions between biologics and azathioprine or methotrexate have been described, we repeated the analysis excluding patients on combined therapies in samples with an absence of serum anti-drug antibodies ($n=24$) obtaining comparable results (Figure 3D).

As stated above, three patients out 30 with Infliximab (10%) and 1 patient out of 22 with Adalimumab (4.5%) showed the presence of anti-drug antibodies (see methods) and were excluded from the analyses presented in Figures 3C and 3D. None of these

patients showed the presence of bactDNA in blood. Two of them had a wtNOD2/wtATG16L1 genotype and were on combined therapy. One patient had a wtNOD2/wtATG16L1 genotype and was on monotherapy with Infliximab. One patient had a wtNOD2/varATG16L1 genotype and was on monotherapy with Adalimumab.

Free anti-TNF- α levels are decreased in the supernatants of neutrophils from variant NOD2/ATG16L1 patients exposed to bactDNA.

We designed an *in vitro* experiment with neutrophils isolated immediately before anti-TNF-alpha infusion (trough levels) from the same 12-patient genotype-stratified subgroups without bactDNA, excluding those that showed the presence of anti-drug antibodies. Results are shown in Figure 4.

Levels of TNF- α were significantly increased after 24-hour *E. coli* bactDNA stimulation in the subgroups bearing a variant vs. wild-type NOD2 genotype (Figure 4A). Baseline and 24-hour unstimulated supernatant levels are also represented. No differences in the apoptotic activity of neutrophils at this time-point were observed among groups (Figure 4B). Forty-eight hours after anti-TNF- α addition, levels of TNF- α were significantly decreased in all subgroups with wild-type NOD2. This reduction was not observed in the supernatants of cells with a NOD2 variant. On the other hand, a significant reduction in free anti-TNF- α levels was found in the supernatants of the subgroups carrying a variant NOD2 genotype, either alone or combined, compared with wild-type NOD2 (Figure 4C). No differences in apoptotic activity of neutrophils between groups were observed at this time-point either (Figure 4D).

Discussion

Patients bearing a NOD2 variant genotype, either alone or combined with an ATG16L1 variant genotype, show an increase in secreted TNF-alpha levels in response to bactDNA translocation and, therefore, a faster consumption of free anti-TNF-alpha. From a clinical perspective, this may lead to a significantly higher percentage of patients requiring an intensified anti-TNF-alpha therapy among variant NOD2/ATG16L1 genotypes as compared to wild-type. This may contribute to the identification of a subgroup of patients that not only require more aggressive therapies to control inflammation but also have an increased risk of bactDNA translocation and, most likely, an increased risk of relapse.

The present study further indicates that the grade of disease activity in CD patients is associated with an increased rate of bactDNA translocation, which in turn is significantly associated with a single or, especially, with a combined NOD2/ATG16L1-variant genotype. The increased pro-inflammatory response observed in patients with translocated bactDNA is further complicated by the impaired phagocytic and bactericidal activities of neutrophils from patients carrying NOD2 gene variants. This seems to further affect the efficacy of anti-TNF-alpha therapies. These results are in keeping with several previous reports linking NOD2 variants with higher risk of complicated CD (27-29)

The number of IBD-associated susceptibility genes has increased in recent years to over 100 at present (16;30). Among them, autophagy-related genes, especially ATG16L1, have emerged as new key players (15;17) along with others that were previously identified, such as NOD2, classically associated with the development of CD (26). However, none of them has been linked so far with the risk of disease flare-ups. Although the association between these gene variants and disease activity was not found in our study, they were significantly associated, either alone or combined, with the presence of bactDNA in blood of CD patients and this fact was in turn significantly associated with disease activity. These results suggest that bactDNA translocation might be facilitated in patients with variant NOD2/ATG16L1-combined genotypes increasing the risk of flare in these patients, as shown in Figure 1. In fact, genetic interactions between polymorphisms in toll-like receptor (TLR)-9, the specific receptor for immunogenic CpG motifs present in bactDNA and CD-associated variants in NOD2 and other genes, have been previously described (31).

In our study, patients bearing a NOD2 gene variant, either alone or combined with a variant ATG16L1 genotype, showed significantly reduced phagocytic and bactericidal activity (Figure 2). NOD2 genetic alterations have also been associated with a defective innate response in the past (32). Interestingly, our data indicates that NOD2 may be involved in the phagocytic and bactericidal capacities of blood neutrophils, facilitating its ability to clear bacteria and their components from the blood of these patients. We hypothesized that the persistence of bactDNA fragments in the blood of NOD2-mutated patients would correlate with an increased pro-inflammatory cytokine response. Accordingly, in addition to the increased pro-inflammatory response to bactDNA presence in wild-type patients, which is in line with previous results in this and other settings (8;33), data shown in Table 4 revealed that NOD2 mutated genotypes were associated with further increments in the concentration of these cytokines.

The established efficacy of anti-TNF- α therapies in CD (34-37) has supported the role of TNF- α as a key player in CD immunopathogenesis. Our results led us to investigate the potential clinical effect of different NOD2/ATG16L1 genotypes and bactDNA presence on the inflammatory response in CD patients receiving treatment with biologics. The proportion of patients on intensified treatment was significantly higher in patients with a variant NOD2/ATG16L1-combined genotype. At the same time, free anti-TNF- α levels were significantly decreased in the serum of patients bearing any of the NOD2 variants and especially in those with a combined NOD2/ATG16L1 variant genotype. As the presence of anti-drug antibodies is a well-known factor affecting drug levels, the analyses were performed considering only patients without serum anti-drug antibodies. These data are clinically relevant since free anti-TNF- α levels represent the available drug in the blood of patients. Low serum anti-TNF- α levels have been associated with a lack or loss of response and therapeutic drug monitoring has been suggested as a useful tool in the clinical management of CD patients (38-40). Inter-individual variability has been a constant flaw in studies reporting pharmacokinetic data on anti-TNF- α levels so far. The use of concomitant drugs such as azathioprine or methotrexate has also been reported to influence clearance of anti-TNF- α and the development of immunogenicity (41;42). We also analysed the subgroup of patients exclusively on anti-TNF- α monotherapy. In our series, results were similar although variability was reduced in patients on monotherapy. These

results suggest that not only combined therapies but also NOD2/ATG16L1 genotypes and the presence of bactDNA may account for the high variability observed in serum free anti-TNF- α levels and might provide an explanation for the need for intensified anti-TNF- α therapy. One may think of the intensified biological schedule as the cause for the increased bacterial translocation and the subsequent bactDNA detection in the varNOD2 group. However, only 20% of patients on anti-TNF- α therapy have bactDNA and, of those, 50% were intensified. This percentage was not significantly different from the 33% of patients without bactDNA who were on drug intensification. Besides, if that were the case, lower bactDNA rates might be expected in varNOD2 patients without vs. with biological therapy but bactDNA is present in 52.7% of varNOD2 patients without vs. 29.4% of varNOD2 patients with anti-TNF- α therapy ($p=ns$).

To further support our assumption, we designed an *in vitro* experiment on neutrophils obtained from patients without bactDNA carrying different genotypes at the time of anti-TNF- α drug infusion (trough level). After bactDNA stimulation and addition of a known Infliximab dose, free drug levels were significantly lower in 72-hour supernatants of cells from patients bearing a double-mutated genotype, suggesting an accelerated drug consumption rate in these patients. Although the highest TNF- α levels were observed in samples from mutated genotypes treated with *E. coli* DNA and despite the fact that 24-hour unstimulated cells didn't show an increase in TNF- α compared to baseline, the possibility of an inflammatory peak, earlier to or independent of BT, is not completely ruled out. Nevertheless, these results point to the identification of a subgroup of CD patients with the potential need for a more aggressive therapeutic approach to efficiently control their disease course or, at least, in which the current therapies are not as effective as in patients with a wild-type NOD2 genotype. In fact, studies have already been conducted to evaluate "top-down" vs. "step-up" therapies and these strategies are presently under discussion (43;44). Besides, the presence of bactDNA was significantly associated in our series with relapse in the 6-month follow-up period. Although evaluating risks factors for relapse was not the aim of this study, the preliminary data reported here supports the design and development of new specifically designed studies including multivariate analysis of known risk factors for the clinical course of the disease such as the lack of mucosal healing (45), the use of non-steroidal anti-inflammatory drugs (NSAIDs) (46-48), treatment withdrawal (49;50) and others.

In summary, combined NOD2/ATG16L1 variant genotypes identify a patient subgroup with an increased risk of bactDNA translocation. This fact may, in turn, exacerbate the inflammatory response and is directly associated with disease activity and the risk of relapse in the short-term. This persistent inflammatory environment would favour the requirement for therapy intensification in mutated patients on treatment with biologics, who would probably be candidates for a different therapeutic strategy.

Reference List

- (1) Hou JK, El-Serag H, Thirumurthi S. Distribution and manifestations of inflammatory bowel disease in Asians, Hispanics, and African Americans: a systematic review. *Am J Gastroenterol* 2009;**104**(8):2100-9.
- (2) Shanahan F, Bernstein CN. The evolving epidemiology of inflammatory bowel disease. *Curr Opin Gastroenterol* 2009;**25**(4):301-5.
- (3) Elson CO, Cong Y, McCracken VJ *et al.* Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* 2005;**206**:260-76.
- (4) Obermeier F, Dunger N, Strauch UG *et al.* CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation. *Gastroenterology* 2005;**129**(3):913-27.
- (5) Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 2002;**347**(6):417-29.
- (6) Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am J Gastroenterol* 1997;**92**(12 Suppl):5S-11S.
- (7) Gutierrez A, Frances R, Amoros A *et al.* Cytokine association with bacterial DNA in serum of patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2009;**15**(4):508-14.
- (8) Gutierrez A, Holler E, Zapater P *et al.* Antimicrobial peptide response to blood translocation of bacterial DNA in Crohn's disease is affected by NOD2/CARD15 genotype. *Inflamm Bowel Dis* 2010.
- (9) Girardin SE, Boneca IG, Viala J *et al.* Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003;**278**(11):8869-72.
- (10) Inohara N, Ogura Y, Fontalba A *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 2003;**278**(8):5509-12.
- (11) Ogura Y, Inohara N, Benito A *et al.* Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 2001;**276**(7):4812-8.
- (12) Ogura Y, Bonen DK, Inohara N *et al.* A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;**411**(6837):603-6.
- (13) Franke A, McGovern DP, Barrett JC *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010;**42**(12):1118-25.
- (14) Lees CW, Barrett JC, Parkes M *et al.* New IBD genetics: common pathways with other diseases. *Gut* 2011;**60**(12):1739-53.

- (15) Hampe J, Franke A, Rosenstiel P *et al.* A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 2007;**39**(2):207-11.
- (16) Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy* 2007;**3**(6):649-51.
- (17) Rioux JD, Xavier RJ, Taylor KD *et al.* Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 2007;**39**(5):596-604.
- (18) Schmid D, Munz C. Innate and adaptive immunity through autophagy. *Immunity* 2007;**27**(1):11-21.
- (19) Kuballa P, Huett A, Rioux JD *et al.* Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS One* 2008;**3**(10):e3391.
- (20) Homer CR, Richmond AL, Rebert NA *et al.* ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology* 2010;**139**(5):1630-41, 1641.
- (21) Sanjuan MA, Dillon CP, Tait SW *et al.* Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 2007;**450**(7173):1253-7.
- (22) Sanjuan MA, Green DR. Eating for good health: linking autophagy and phagocytosis in host defense. *Autophagy* 2008;**4**(5):607-11.
- (23) Sands BE. From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology* 2004;**126**(6):1518-32.
- (24) Silverberg MS, Satsangi J, Ahmad T *et al.* Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005;**19 Suppl A**:5-36.
- (25) Such J, Frances R, Munoz C *et al.* Detection and identification of bacterial DNA in patients with cirrhosis and culture-negative, nonneutrocytic ascites. *Hepatology* 2002;**36**(1):135-41.
- (26) Hampe J, Grebe J, Nikolaus S *et al.* Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 2002;**359**(9318):1661-5.
- (27) Ahmad T, Armuzzi A, Bunce M *et al.* The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 2002;**122**(4):854-66.
- (28) Economou M, Trikalinos TA, Loizou KT *et al.* Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol* 2004;**99**(12):2393-404.

- (29) Radlmayr M, Torok HP, Martin K *et al.* The c-insertion mutation of the NOD2 gene is associated with fistulizing and fibrostenotic phenotypes in Crohn's disease. *Gastroenterology* 2002;**122**(7):2091-2.
- (30) Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;**447**(7145):661-78.
- (31) Torok HP, Glas J, Endres I *et al.* Epistasis between Toll-like receptor-9 polymorphisms and variants in NOD2 and IL23R modulates susceptibility to Crohn's disease. *Am J Gastroenterol* 2009;**104**(7):1723-33.
- (32) van Heel DA, Ghosh S, Butler M *et al.* Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 2005;**365**(9473):1794-6.
- (33) Frances R, Zapater P, Gonzalez-Navajas JM *et al.* Bacterial DNA in patients with cirrhosis and noninfected ascites mimics the soluble immune response established in patients with spontaneous bacterial peritonitis. *Hepatology* 2008;**47**(3):978-85.
- (34) Hanauer SB, Feagan BG, Lichtenstein GR *et al.* Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002;**359**(9317):1541-9.
- (35) Sands BE, Anderson FH, Bernstein CN *et al.* Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004;**350**(9):876-85.
- (36) Rutgeerts P, D'Haens G, Targan S *et al.* Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999;**117**(4):761-9.
- (37) Present DH, Rutgeerts P, Targan S *et al.* Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999;**340**(18):1398-405.
- (38) Afif W, Loftus EV, Jr., Faubion WA *et al.* Clinical utility of measuring infliximab and human anti-chimeric antibody concentrations in patients with inflammatory bowel disease. *Am J Gastroenterol* 2010;**105**(5):1133-9.
- (39) Bendtzen K, Ainsworth M, Steenholdt C *et al.* Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. *Scand J Gastroenterol* 2009;**44**(7):774-81.
- (40) Colombel JF, Feagan BG, Sandborn WJ *et al.* Therapeutic drug monitoring of biologics for inflammatory bowel disease. *Inflamm Bowel Dis* 2012;**18**(2):349-58.
- (41) Mould DR, Green B. Pharmacokinetics and pharmacodynamics of monoclonal antibodies: concepts and lessons for drug development. *BioDrugs* 2010;**24**(1):23-39.
- (42) Colombel JF, Sandborn WJ, Reinisch W *et al.* Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010;**362**(15):1383-95.

- (43) Burger D, Travis S. Conventional medical management of inflammatory bowel disease. *Gastroenterology* 2011;**140**(6):1827-37.
- (44) Lin MV, Blonski W, Lichtenstein GR. What is the optimal therapy for Crohn's disease: step-up or top-down? *Expert Rev Gastroenterol Hepatol* 2010;**4**(2):167-80.
- (45) De CP, Kamm MA, Prideaux L *et al.* Mucosal healing in Crohn's disease: A systematic review. *Inflamm Bowel Dis* 2012.
- (46) Bjarnason I, Hayllar J, MacPherson AJ *et al.* Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* 1993;**104**(6):1832-47.
- (47) Forrest K, Symmons D, Foster P. Systematic review: is ingestion of paracetamol or non-steroidal anti-inflammatory drugs associated with exacerbations of inflammatory bowel disease? *Aliment Pharmacol Ther* 2004;**20**(10):1035-43.
- (48) O'Brien J. Nonsteroidal anti-inflammatory drugs in patients with inflammatory bowel disease. *Am J Gastroenterol* 2000;**95**(8):1859-61.
- (49) Rutgeerts P, Feagan BG, Lichtenstein GR *et al.* Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004;**126**(2):402-13.
- (50) Rutgeerts P, Diamond RH, Bala M *et al.* Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006;**63**(3):433-42.

Figure legends.

Figure 1. (A) Distribution of NOD2/ATG16L1-combined genotypes in CD patients stratified by CDAI. (B) Percentage of bactDNA presence in each studied genotype distributed by CDAI; * $p < 0.01$ compared with the wtNOD2/wtATG16L1 genotype group. CDAI: Crohn's disease activity index; bactDNA: bacterial DNA; wt: wild-type; var: variant.

Figure 2. Phagocytic (A) and bactericidal (B) activities in neutrophils of patients with NOD2 / ATG16L1 combined genotypes (n=12/each group) without bactDNA translocation into blood; * $p < 0.01$ compared with wtNOD2 groups.

Figure 3. (A) Percentage of patients on intensified anti-TNF- α therapy stratified according to NOD2/ATG16L1-combined genotypes. (B) Percentage of patients on intensified anti-TNF- α therapy according to the presence of bactDNA. (C) Free anti-TNF- α levels in serum of patients receiving therapy with biologics, either alone or combined with immunosuppressive treatment, stratified according to each genotype studied as well as to the presence of bactDNA; * $p < 0.01$ compared with wtNOD2 groups. (D) Free anti-TNF- α levels in serum of patients on monotherapy with biologics, stratified according to each genotype studied and to the presence of bactDNA; * $p < 0.01$ compared with wtNOD2 groups.

Figure 4. (A) TNF- α levels in the supernatants of neutrophils from patients with different NOD2/ATG16L1 genotypes exposed to *E. coli*-DNA; \$ $p < 0.01$ compared with baseline and unstimulated conditions; * $p < 0.01$ compared with wtNOD2 groups. (B) Apoptotic activity of 24-hour cultured neutrophils from patients of each genotype subgroup. (C) Free anti-TNF- α levels in the supernatants of neutrophils from patients with studied NOD2/ATG16L1 genotypes exposed to *E. coli*-DNA and treated with Infliximab; * $p < 0.01$ compared with wtNOD2 groups. (D) Apoptotic activity of 72-hour cultured neutrophils from patients of each genotype subgroup.

Table 1. Patient characteristics.

	CDA Index	
	<150 (n=136)	>150 (n=43)
Age (years)	40 ± 14	42 ± 15
Weight (kg)	70,12 ± 15,76	68,88 ± 20,78
Genre (male/female)	65/71	18/24
Smoking habit n (%)	48 (35%)	14 (32%)
Disease duration (months)	112,52 ± 91,66	106,54 ± 98,19
Resection n (%)	36 (26%)	13 (30%)
Montreal A (age of onset) n (%)		
A1 (<=16)	6 (4,4%)	3 (7%)
A2 (17-40)	107 (78,6%)	30 (69,7%)
A3 (>40)	23 (17%)	10 (23,3%)
Montreal L (location) n (%)		
L1 (ileal)	70 (51,4%)	23 (53,4%)
L2 (colonic)	32 (23,5%)	5 (11,6%)
L3 (ileocolonic)	31 (22,8%)	12 (28%)
L4 (upper isolated)	3 (2,2%)	3 (7%)
Montreal B (behaviour) n(%)		
B1 (non-stricturing, non-penetrating)	68 (50%)	19 (44,2%)
B1p (non-stricturing, non-penetrating, penetrating perianal disease)	11 (8%)	2 (4,6%)
B2 (stricturing)	22 (16,3%)	13 (30,2%)
B2p (stricturing, perianal disease associated)	7 (5,1%)	-
B3 (penetrating)	24 (17,6%)	5 (13,6%)
B3p (penetrating, penetrating perianal disease)	4 (2,9%)	4 (9,3%)
Therapy n (%)		
Mesalazine	35 (25,7%)	5 (11,6%) *
Steroids	-	2 (4,6%)
Azathioprine	49 (36,0%)	4 (9,3%) *
Infliximab	7 (5,1%)	5 (11,6%)
Adalimumab	12 (8,8%)	2 (4,6%)
Metotrexate	-	1 (2,4%)
Mesalazine and Steroids	3 (2,2%)	5 (11,6%)
Mesalazine and Azathioprine	7 (5,1%)	2 (4,6%)
Azathioprine and Steroids	2 (1,4%)	10 (23,2%) *
Infliximab and Azathioprine	8 (5,8%)	4 (9,3%)
Adalimumab and Azathioprine	5 (3,6%)	-
Infliximab and Metotrexate	2 (1,4%)	-
Infliximab and Steroids	1 (0,7%)	2 (4,6%)
Adalimumab and Steroids	3 (2,2%)	-
Infliximab and Azathioprine and Steroids	-	1 (2,3%)
No treatment	2 (1,4%)	-
ESR (mm)	19.4 ± 16.3	40.5 ± 27.4 *
CRP (mg/dL)	0,53 ± 1,04	3,43 ± 4,99 *
Hemoglobin (g/dL)	13,93 ± 3,02	12,49 ± 1,66 *
Albumin (mg/dL)	4073,05 ± 453,02	3588,2 ± 573,47 *
Total WBCs (mm3)	6907,7 ± 2757,24	7725,02 ± 2642,26
Temperature (°C)	36,09 ± 0,26	36,54 ± 0,58 *
Pulse rate (bpm)	70,17 ± 5,43	72 ± 7,47

All values shown as mean ± standard deviation or percentage. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; * p<0.05 when compared with CDA index<150

Table 2. Distribution of genotypes and allelic frequencies in CD patients and controls.

	Genotype [n (%) patients / controls]			Variant allele frequency (%)
	Homozygous wild-type	Heterozygous	Homozygous variant	
NOD2 SNP8 (R702W)	136 (76%) / 24 (96%)	43 (24%) / 1 (4%)	0 / 0	12.0 / 2.0
NOD2 SNP12 (G908R)	152 (85%) / 24 (96%)	26 (14.5%) / 1 (4%)	1 (0.5%) / 0	7.8 / 2.0
NOD2 SNP13 (L1007finsC)	164 (92%) / 24 (96%)	15 (8.4%) / 1 (4%)	0 / 0	4.7 / 2.0
ATG16L1 (T300A)	73 (41%) / 22 (88%)	84 (47%) / 5 (20%)	22 (12.3%) / 0	35.8 / 10.0

Table 3. Multivariate analyses on disease activity (A), bacterial DNA (B) and relapse at 6 months (C) including variables with a *p* value < 0.1 on univariate analysis.

A)

Dependent variable: Disease activity (CDAI < 150/CDAI > 150)

Multivariate analysis

Variable	P	OR	95%CI
Presence of bacterial DNA fragments (yes/no)	0.008	7.422	1.690 – 32.596
Steroids treatment (yes/no)	0.001	27.303	4,233 – 176,116
Infliximab treatment (yes/no)	0.001	16.649	3.213 – 86.288
C-reactive protein (mg/dL)	0.001	3.833	1.679 – 8.752

B)

Dependent variable: bacterial DNA

Multivariate analysis

Variable	P	OR	95%CI
Disease activity (CDAI > 150/CDAI < 150)	0.001	5.084	1.972 – 13.106
Genotype			
wtNOD2/wtATG16L1	0.000		
varNOD2/wtATG16L1	0.020	5.117	1.287 – 20.346
wtNOD2/varATG16L1	0.0401	2.221	1.394 – 5.118
varNOD2/varATG16L1	0.000	12.728	3.738 – 43.344

C)

Dependent variable: Relapse at 6 months (yes/no)

Multivariate analysis

Variable	P	OR	95%CI
Presence of bacterial DNA fragments (yes/no)	0.003	5.132	1.764 – 14.926

Table 4. Serum cytokine levels in patients with different NOD2/ATG16L1-combined genotypes according to the presence of bactDNA.

		TFN-alpha (pg/mL)	IFN-gamma (pg/mL)	IL-12 (pg/mL)
BactDNA - (n=128)	wtNOD2 / wtATG16L1 (n=43)	28,08 ± 6,89	30,21 ± 4,06	54,19 ± 7,70
	varNOD2 / wtATG16L1 (n=18)	28,71 ± 4,91	31,81 ± 4,37	52,31 ± 6,45
	wtNOD2 / varATG16L1 (n=48)	30,09 ± 4,50	34,78 ± 7,64	52,64 ± 5,71
	varNOD2 / varATG16L1 (n=19)	30,12 ± 3,48	32,04 ± 5,70	50,72 ± 5,20
BactDNA + (n=51)	wtNOD2 / wtATG16L1 (n=5)	49,57 ± 6,92 *	59,32 ± 6,1 *	91,16 ± 11,8 *
	varNOD2 / wtATG16L1 (n=8)	75,67 ± 7,80 * [†]	82,86 ± 7,48 * [†]	124,01 ± 15,5 * [†]
	wtNOD2 / varATG16L1 (n=9)	50,70 ± 8,73 *	61,43 ± 6,6 *	98,73 ± 12,43 *
	varNOD2 / varATG16L1 (n=29)	79,50 ± 8,93 * [†]	85,60 ± 8,27 * [†]	138,49 ± 17,5 * [†]

* $p < 0.01$ compared with same genotype in bactDNA negative patients;

[†] $p < 0.01$ compared with the wtNOD2 genotypes in bactDNA positive patients.